VIABILITY AND FERTILITY OF DROSOPHILA EXPOSED TO SUB-ZERO TEMPERATURES[1]

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Introduction

The dependence of the effects of x-irradiation on various physical variables has provided an effective tool bearing on the problem of chromosome breakage and gene mutation induced by such irradiation. The study of one of these variables, temperature, is obviously limited to the range which the organism can survive. This is particularly true of a large multicellular animal such as Drosophila. Since in general low temperatures do not have detrimental biological effects unless ice crystals are produced and since the freezing point of many cells is far below what would be predicted from their osmotic pressure (Luyet and Gehenio, 1940), it seemed likely that one could extend the temperature range of experiments on Drosophila most effectively by exploring the effects of temperatures lower than 0° C. The design of the experiments to be described was dictated by the conditions of the typical x-ray treatment; certain aspects of the broader problem of viability and fertility under these conditions have therefore been emphasized, others ignored.

Materials and Methods

Equipment

Treatment was made in a specially designed cold temperature chamber, consisting of an insulated box of about two cubic foot capacity cooled by coils from a ⅓-horsepower refrigeration unit and heated simply by a hundred watt lamp. A partition divided the chamber into two sections, the upper of which was used for treatment, while the lower contained the heating and cooling units. A $\frac{1}{70}$ horsepower blower at one end of the partition forced the air from the lower into the upper chamber; an opening over the refrigerator coils at the other end provided for the free circulation of the air. A thermostat was placed in the blower air blast from the lower section into the upper one. Thus a heating-cooling cycle was completed about every half minute. Thermocouple measurements showed that the air in the upper chamber varied during the cycles not more than 1½° C. on either side of the average temperature measured with a standard mercury thermometer. In those cases where the temperature variation was to be minimized the material was placed in a 15 cubic inch cardboard box which could be closed after the desired temperature had been reached in the chamber. The variations in the cardboard box, again meas-

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ured with a thermocouple, amounted to only ten per cent of those in the surrounding air blast. Cellophane windows were provided in both the removable cork top of the chamber and in the small box to facilitate checking the material during treatment and to minimize the absorption of X-rays during irradiation.

Rate of change of internal temperature

Thermocouple measurements inside the thorax of the fly subjected to an air blast at − 8° C. indicate that the internal temperature of the fly drops at an initial rate of about 1.6° C. per second, the rate becoming less as the gradient between the external and internal temperatures decreases, and that the fly and air temperatures are the same in from two to three minutes. The authors are indebted to Professor A. C. Fabergé who constructed by intricate plating techniques a very fine thermocouple for the purpose of making these measurements.

Genetic methods

The choice of stocks used in this work was determined by the plan of concurrent irradiation experiments; therefore the highly inbred Canton-S strain of *Drosophila melanogaster* was used. In fertility tests, these flies were provided with mates from the "Muller-5" strain which is now widely used in tests of irradiation effects in this species. The individuals to be treated were placed in size 00 gelatin capsules with holes punctured in both ends for rapid ventilation. Usually twenty flies at a time were placed in one capsule; numbers presented in the viability experiments therefore occur in approximate multiples of twenty which represent grouping of individual runs. Tests of fertility were made by placing single treated individuals with appropriate mates in 8 dram shell vials containing standard cornmeal-molasses-agar Drosophila culture medium.

Results

General behavior at low temperatures

As the internal temperature of the fly drops, it becomes sluggish and at + 3° C. all movement stops. A normal posture is then maintained regardless of the extent of the subsequent decrease in temperature. Lethality manifests itself during the period of recovery from the cold treatment. If the treatment is too severe, the fly assumes a posture characteristic of death by overetherization, i.e. the wings are held parallel in an upwards position. In some instances sub-lethal temperatures have impaired the locomotor control of the flies. Such individuals, after removal to room temperature, remain motionless or make feeble and uncoordinated attempts to walk. One such ataxic individual remained alive for 3 days; but for the purpose of this work such cases will be included with the deaths.

Effects of pretreatments

In the earliest series it became obvious that Drosophila under the influence of ether were particularly sensitive to the cold shocks. In the experiments to be described, the individuals were always allowed to recover completely from etherization before treatment. The high sensitivity under these conditions may account for the

failure of some workers (see the discussion) to use temperatures below 0° C. Other techniques were tried to increase the resistance to cold without effect; these include pretreatment with $CaSO_4$ desiccant, with temperatures less severe than the final one (1° C. for 20 minutes before exposing the fly to − 10° C.), and with a sudden exposure of the animals to a temperature lower (− 20° C.) than that of treatment.

Viability of Drosophila at low temperatures

At 0° C., Drosophila males can survive for about 24 hours; since they die in about the same time in isolated capsules at room temperature, no additional work

TABLE I

Mortality of Drosophila melanogaster males treated for various durations of time at 4 subzero temperatures. A = duration of treatment in minutes; B = number of males treated; C = number killed by treatment; D = percentage of mortality.

− 5° C.														
A	10	20	30	40	50	60	70	80	90	100	110	120		
B	20	40	60	40	20	60	80	82	80	40	20	40		
C	1	0	1	1	0	3	5	41	54	2	11	30		
D	5	0	1.6	2.5	0	5	6.3	50	68	5	55	75		
− 10° C.														
A	10	20	21	22	23	24	25	26	27	28	29	30	40	50
B	60	178	60	60	20	60	20	60	20	60	20	237	80	20
C	6	27	1	1	2	22	20	59	20	60	20	228	80	20
D	10	15.2	1.6	1.6	10	36.7	100	98.4	100	100	100	96.2	100	100
− 15° C.														
A	1	2	3	4	5	6	7	8	9	10	11	12	13	13
B	20	20	20	20	40	20	40	40	20	20	20	20	20	180
C	1	7	9	4	16	3	15	23	16	14	17	13	15	180
D	5	35	45	20	40	15	38	58	80	70	85	65	75	100
− 20° C.														
A	.5	1	1.5	2										
B	20	20	20	20										
C	2	13	16	20										
D	10	65	80	100										

has been done in this range. The percentage of mortality for various durations of exposure at − 5° C., − 10° C., − 15° C. and − 20° C. are given in Table I. At − 5° C., two hours duration does not kill 100 per cent of the flies; at − 10° C., 25 minutes treatment is completely lethal; at − 15° C., 14 minutes is lethal and at − 20° C., 2 minutes. The thermocouple measurements previously referred to suggest that in all series except the last, the time required for the flies to reach the temperature of the chamber is insignificant; in the last, however, some temperature between − 15° C. and − 20° C. is lethal per se without respect to time duration. One set at − 10° C. run at 2 minute intervals from 14 to 32 minutes, with parallel sets of males and females showed identical sensitivities of the two sexes.

The sterilization of fertilized females

Preliminary tests of 32 females subjected to − 10° C. for one-half hour and subsequently mated had shown their fertility to be unimpaired. On the other hand, about 200 fertilized females exposed to − 5° for 56 minutes during an irradiation experiment proved to be sterile. Since, in the latter case, mature sperm as well as ova were subjected to the treatment, it appeared likely that the sterility was caused by an inactivation of the sperm stored in the female, or "desemination" (Muller, 1944). A number of different kinds of tests were made to determine whether the adverse effect of the low temperatures was on the ova or sperm carried by the fertilized females. In these runs, Canton-S females were placed in quarter pint milk bottles with Canton-S males for three days or longer in order to insure the insemination of most of them, the proportion fertilized being determined by a test of a sample of them made as a control. After treatment, they were placed indi-

TABLE II

Fertility of fertilized Drosophila females exposed to −5° C. and −10° C. for varying durations of time

Duration	Females treated	Females fertile	Offspring per fertile female	Females producing only one offspring
Temperature = −5° C.				
0	25	24	79	0
15	50	1	88	11
30	50	1	88	10
45	50	2	43	13*
60	50	1	100	8
75	50	0	—	8
90	50	0	—	6
Temperature = −10° C.				
0	25	24	92	0
5	50	0	—	12
10	36	0	—	4
15	50	0	—	6
20	50	0	—	5

* Includes one female which gave 2 offspring and another which gave 4.

vidually in shell vials with food, and their offspring counted nineteen days later, unless otherwise noted.

Out of 50 fertilized females exposed to 0° C. for 6 hours, 7 were sterile; the untreated control showed 4 sterile out of 50. This treatment is ineffective in sterilization. A larger series was subjected to − 5° C. for periods from 0 to 90 minutes in 15 minute intervals and to − 10° C. for periods from 0 to 20 minutes in 5 minute intervals. The 0 minute series in each case represent the controls. The results are shown in Table II. It is obvious that − 5° C. does sterilize the females since only 5 out of 300 treated were fertile, whereas 24 out of 25 in the controls were fertile. The five cases of fertility after treatment at − 5° C. with durations from 15 to 60 minutes were cases of complete fertility. When the exposures were longer than 60 minutes, no completely fertile females were found. Likewise exposures to − 10° C. sharply decrease the percentage of fertile females. The

sporadic production of single, or very few, offspring by treated females noted in Table II was probably overlooked in the earlier runs.

In order to determine whether there is any effect of the cold shocks in the germ line of the female, which would account for the above results, a series was run in which fertilized Canton-S females were mated, after exposure to a sterilizing dose, to Muller-5 males. In this way it is possible to differentiate between sperm stored in the female at the time of treatment which would produce round-eyed females progeny, and the sperm introduced by the Muller-5 males after treatment, which would give narrow-eyed female offspring. In the event of an effect on the germ line of the female, no offspring of either type would be anticipated. Progeny counts were made 13 days after treatment; this short egg-laying time should make more obvious any temporary sterilization of the female which a longer egg-laying period might obscure. The results are shown in Table III. The single female which produced after treatment offspring of the first insemination yielded only one, like the sporadic cases described above. The fertility of females treated and subsequently mated is, in this run, higher than that of the untreated females not mated afterwards. This difference may be due to an increased viability of offspring of the second mating. However, the essential point is that the germ cells of the female are apparently not affected by the treatment and that such sterilization as does occur must be attributed to the killing of sperm stored in the female.

TABLE III

The productivity of fertilized females exposed to −10° C. for 20 minutes and subsequently mated to Muller-5 males compared with that of females so treated but not mated subsequently and with untreated and unmated females

	Untreated not mated	Treated unmated	Treated mated
Total ♀ ♀ treated	23	11	79
♀ ♀ producing offspring of first insemination	11	0	1
♀ ♀ producing offspring of second insemination	0	0	47
Average no. of ♀ ♀ offspring per fertile female	18.6	0	33.5

Dissections of deseminated females and their untreated sisters as controls revealed no motile sperm in the ventral receptacles or spermathecae of the former, although there was an abundance of motile sperm in both these organs of the controls. In addition, the quantity of sperm (immotile) in the ventral receptacles of the deseminated females was much smaller than that in the controls, in most cases the receptacles appearing completely empty as if a contraction had expelled the sperm. The natural striations of the chitinous spermathecal wall prevented any comparisons of quantity in the two sets, although in a few cases where a spermatheca of a treated female had been broken by pressure, sperm appeared in approximately normal quantity; they were, however, immotile.

The sporadic occurrence of one or two offspring among deseminated females may have one of two explanations. Either the treatment is not inactivating all the stored sperm, or those few offspring result from eggs already fertilized, or in the process of fertilization, at the time of treatment. These alternatives have been dif-

ferentiated in two ways. First if exposure to a deseminating dose kills all but small fraction of the stored sperm, then the application of two such doses should be effective in decreasing their incidence even more. Fifty fertilized females, subjected to two sterilizing doses of − 10° for 15 minutes separated by an interval of 2 hours at room temperature, produced 8 offspring, each from one treated female. Twenty-five untreated controls produced an average of 109.7 offspring in 24 fertile vials. This frequency of sporadics is of the same order of magnitude as that in the previous single shock treatments.

On the other hand, if the sporadic cases are to be accounted for by the presence of fertilized eggs in the oviduct of the female at the same time of treatment, then, since those eggs are laid first, the sporadic individuals should come primarily from the first eggs deposited. Once again 50 Canton-S females, presumably fertile, were treated with − 10° for 15 minutes and transferred to new culture bottles on 4 successive days. The eggs laid on the first day included 14 sporadic cases; those on the second, third and fourth, none. A similar run, interrupted after the second day, gave 8 sporadic cases in the first day, none on the second. The controls in both the above cases were highly fertile. It seems reasonable to conclude, then, that these occasional single progeny appearing after the cold treatments result from eggs which had been fertilized before the time of treatment.

Effects on fertility of the male

In marked contrast to the pronounced lethality of cold shocks on sperm stored in female Drosophila, spermatozoa in the males are more resistant to changes in temperature, although here, too, there appears to be some lethality. Thirty-eight males exposed to −10° C. for twenty minutes were all fertile. Their offspring appeared in the customary ten day period, which contradicts the possibility that the mature sperm were killed and that sperm differentiating after treatment were used.

TABLE IV

Sterility and productivity of Drosophila males exposed to low temperatures with and without 3600 r. of x-rays during a 56 min. interval

	Irradiated			Unirradiated	
	25° C.	0.5° C.	−5° C.	0.5° C.	−5° C.
Total treated ♂♂	65	102	115	100	100
Total fertile ♂♂	60	48	11	92	50
% fertile ♂♂	92.4	47.1	9.6	92	50
Number of female offspring/fertile male	30.1	11.8	9.4	22.4	22.4

Likewise 74 out of 158 (equals 47 per cent) of males treated with − 5° C. for one hour were fertile whereas a smaller untreated control series showed that 15 out of 19, or 78 per cent, were fertile. In all these cases, those males which produced any offspring at all produced the normal number. Comparable time-temperature series on fertilized females (see above) were almost 100 per cent effective in killing sperm. When males are dissected after treatment with an exposure that kills all the sperm

stored in females, there appears to be no mortality of sperm in the testes or seminal vesicle.

In agreement with the observations of others (Medvedev, 1935; Mickey, 1939) that irradiation at low temperatures decreases the fertility of males to an extent greater than that anticipated on a single additive effect basis, the data in Table IV show the fertility and productivity of males (mated singly to 2 Muller-5 females in shell vials) after exposure to 0.5° C. and − 5° C., with and without a dose of 3600 r during a 56 minute treatment. In the unirradiated series, the percentage of males completely sterilized increases with decreasing temperature, but the number of F_1 female progeny (the males not being counted for technical reasons) from the fertile males is essentially normal under the conditions of the experiment. However, with irradiation not only does the percentage of fertile males drop more rapidly, but the number of female progeny of the fertile males is between a half and a third normal. This is apparently related in part, at least, to the greater production of chromosomal aberrations during irradiation, at the lower temperatures, which will be discussed in more detail elsewhere.

Discussion

Applicability of temperatures below 0° C.

From the results of the experiments described above, it is clear that Drosophila will tolerate somewhat lower temperatures than previous workers have used. Thus, there are a number of accounts in the literature of the use of low temperatures in the range of + 3° C. to + 15° C.; in a few cases 0° C. has been reached (Medvedev, 1935; Papalaschwili, 1935; Mickey, 1939; King, 1947) and there is one instance (Kerkis, 1939) where Drosophila has been subjected to a temperature of − 6° C. It seems clear that temperatures below 0° C. are generally applicable provided that care is taken to insure complete recovery from etherization before, and adequate ventilation during treatment. This may permit a decisive test of the hypothesis that the genetic effects of x-irradiation are the immediate result of ionization, since this hypothesis predicts that the results should be temperature independent.

Desemination of Drosophila females

In many types of experiments with Drosophila, one of the most burdensome chores is the collection of virgin females. The observation that sperm stored in a female may be killed by the application of low temperatures, without affecting the fertility of the treated females in subsequent matings provides an effective tool in Drosophila work. Briefly summarized the procedure adopted for this treatment is the following: From 50 to 100 etherized females are placed in one size 00 gelatin capsule which is ventilated by pin holes at both ends. After an hour or two, during which time the flies recover completely from the etherization, the capsules are placed in a cold air blast of − 10° C. for 10 minutes or of − 5° C. for 90 minutes. Upon removal from the low temperature, they may be mated immediately if their sporadic progeny are distinguishable genetically from those of the post-treatment mating, otherwise they should be kept in a culture bottle for a day before mating to allow them to deposit the few fertilized eggs unaffected by the treatment.

Summary

1. At − 5° C. about 50 per cent mortality of Drosophila melanogaster is reached after two hours; at − 10° C. a 20 minute exposure kills very few whereas a 25 minute exposure is almost completely lethal; at − 15° C. about 50 per cent survive exposures less than 10 minutes long whereas an exposure of 13 minutes or longer is completely lethal; and at − 20° C. all individuals are killed within a few minutes.

2. Cold shocks of air at − 5° C. for 75 to 90 minutes and at − 10° C. for 5 to 20 minutes are lethal to sperm stored in adult females although such treatment has no effect on the subsequent fertility of such females. Males are not sterilized to any great extent by such exposures.

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